Immunity
Review

Intracellular Toll-like Receptors

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DOI 10.1016/j.immuni.2010.03.012

Foreign nucleic acids, the signature of invading viruses and certain bacteria, are sensed intracellularly. The nucleic acid-specific Toll-like receptors (TLRs) detect and signal within endolysosomal compartments, triggering the induction of cytokines essential for the innate immune response. These cytokines include proinflammatory molecules produced mainly by macrophages and conventional dendritic cells, as well as type I interferons, which are produced in great quantities by plasmacytoid dendritic cells. The cellular and molecular pathways by which nucleic acids and TLRs meet within the endosome assure host protection yet also place the host at risk for the development of autoimmunity. Here, we review the latest findings on the intracellular TLRs, with special emphasis on ligand uptake, receptor trafficking, signaling, and regulation.

Introduction

The host resists infection in many different ways but, in one way or another, must always discriminate self from nonself. Such discrimination may depend upon sensing things that are diminished or absent during infection though present under normal conditions. One example is the downregulation of class I MHC molecules during many viral infections, a change that is perceived by NK cells. Self-nonself discrimination may also depend upon seeing concomitants of infection. This includes changes in the intracellular milieu and the expression of molecules indicative of "stress." Microbial proteases may initiate proteolytic cascades that culminate in the production of ligands that initiate an innate immune response (Ferrandon et al., 2007). But perhaps the most basic and broadly applied scheme for self-nonself discrimination depends upon receptors for molecular signatures unique to microbes and broadly represented among microbial taxa. The existence of such signatures has been known for many decades, and just over one decade ago, Toll-like receptors (TLRs) were identified by genetic means as the sensors that detect them. First LPS (Poltorak et al., 1998), then DNA, RNA, lipopeptides, flagellin, and other components of microbes were shown to be TLR ligands. In *Drosophila*, such sensors also exist. These are the GNBPs and PGRPs (Ferrandon et al., 2007). PGRP-SA and two of the GNBPs converge upon Toll, the namesake of the TLRs, and the first member of the family known to participate in microbe sensing (Lemaitre et al., 1996).

Because they are capable of recognizing molecules derived from viruses, fungi, bacteria, and protozoa, the TLRs are able to sense most of the infections we might ever encounter. And their role in sensing is indispensable. The backup provided by other sensors is inadequate to cope with most infections in the absence of TLR signaling. Mice lacking MyD88 and TRIF, which cannot generate signals via any of the TLRs or via the structurally related IL-1, IL-18, and IL-33 receptors rarely survive to weaning age without antibiotic support (Hoebe et al., 2003). Even mutations affecting individual TLRs, such as TLR2 (Takeuchi et al., 2000), TLR4 (Poltorak et al., 1998), or TLR9 (Tabeta et al., 2004), cause quite obvious susceptibility to specific infectious agents.

All the same, there is considerable redundancy in systems that detect microbes. One indication of this is the relative preservation of adaptive immune responses in the absence of TLR signaling. Conventional adjuvants drive strong adaptive immune responses in the absence of TLR signaling (Gavin et al., 2006). Redundancy is also to be seen in the augmentation of adaptive immune responses witnessed during conventionalization of germ-free mice with enteric microbes, which occurs even if TLR signaling is abolished by mutations (Slack et al., 2009). Thus, although TLRs do mediate adjuvant effects, adaptive immune responses can nonetheless occur perfectly well in their absence. It is now well understood that microbes can initiate an adaptive response via several sensing pathways and, in most cases, can do so even if there is no TLR signaling at all.

Another source of redundancy stems from the fact that each species of microbe usually displays multiple TLR ligands in addition to ligands that trigger other classes of innate immune receptors, including the RIG-I-like helicases (RLHs) (Yoneyama and Fujita, 2009), NOD-like receptors (NLRs) (Martinon et al., 2007), or glycan receptors (Lee and Kim, 2007). A microbe that evades one pathway will often be detected by another.

Yet, we provisionally assume that, not only in their ligand specificity but in other characteristics as well, TLRs must each make unique contributions to host resistance as a whole. If they did not, we might expect they would be eliminated by the pressure of random mutation. Here, we suggest that distinct sensing pathways evolved to match the characteristics and lifestyles of divergent microbes, which may be extracellular, cytoplasmic, or resident within endosomal compartments. We further consider that the host has evolved specialized cellular defenses to cope with infection, wherein different cells of the immune system and, indeed, all cells of the host may be endowed with "optimized" systems for the perception of microbes, often microbes with a set of common characteristics.

The TLR family includes receptors residing both at the cell surface and intracellularly, highlighting the specialization of receptor subsets for particular tasks. Many of the unique properties of the intracellular receptors have only recently been deciphered. In this review, we discuss the intracellular TLRs, their ligands, their signaling, mutations that disrupt their function, and their relationship to other receptors that detect infection.

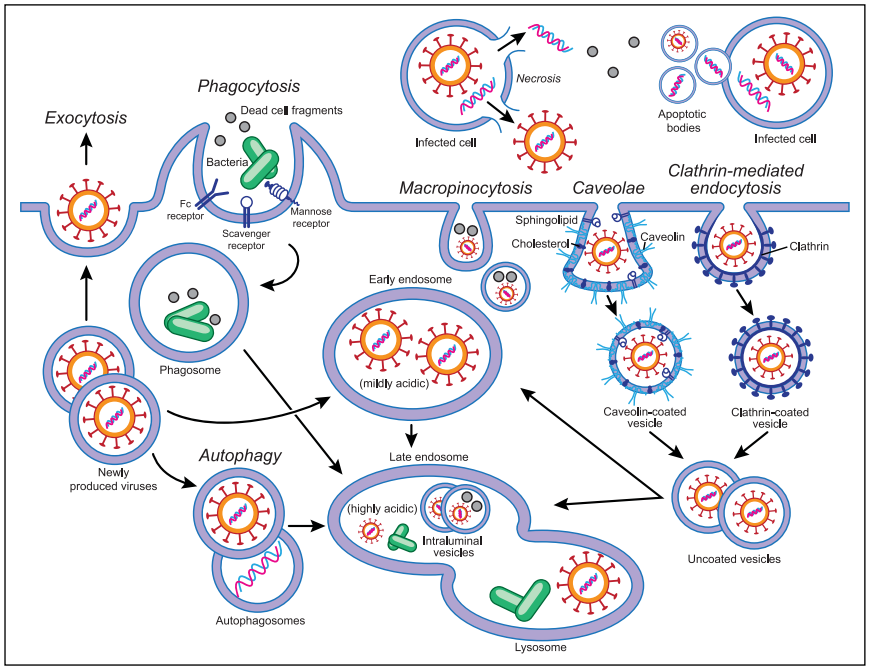

Figure 1. Mechanisms for Internalization of Nonself

The engulfment of solid particles by phagocytosis can mediate uptake of whole bacteria and dead cell fragments and is triggered by specific receptors, including the mannose receptor, Fc receptors, and scavenger receptors. Fluid phase endocytosis, or macropinocytosis, allows uptake of suspended or cell-adherent particles. Receptor-mediated endocytosis via clathrin or caveolin enables absorption of a variety of particles and molecules, including nutrients and pathogens. Ultimately, vesicles formed at the plasma membrane fuse with mildly acidic early or recycling endosomes. Phagosomes may directly fuse with the highly acidic late endosomes or lysosomes. Vesicle cargo is finally delivered to lysosomes through fusion between late endosomes and lysosomes.

Pathways to Recognition: How Cells Internalize Nonself

Cells must survey the extracellular milieu for signs of infection, and in doing so, they run the risk of being infected and killed by microbes. For this reason, they internalize microbes into the endocytic pathway: a hostile environment that is nonetheless capable of detecting infection and reporting it, first to the phagocytic cell and then to other cells of the body. The endocytic pathway accommodates molecules derived from cells and microbes alike. It acquires them through diverse processes that have much in common with one another yet are distinct enough to codify (Figure 1).

Phagocytosis is triggered by specific receptors, including the mannose receptor, complement receptor, Fc receptors, phosphatidylserine receptor, and scavenger receptors. Phagocytic uptake requires actin cytoskeletal reorganization and is critically dependent on Rho family GTPases, allowing protrusions from the membrane to wrap around the target and engulf it (Groves et al., 2008). Ultimately, phagosomes fuse with late endosomes or lysosomes, but phagosome dwell time and the precise trafficking mechanism are dictated by the identity of the uptake receptor and the nature of the cargo (Conner and Schmid, 2003). Some cells are far more active at phagocytosis than others, such as macrophages and polymorphonuclear leukocytes that are highly specialized for the process.

Fluid phase endocytosis, or macropinocytosis, allows uptake of suspended or cell-adherent particles. The mechanisms are similar to phagocytosis in that actin cytoskeletal rearrangement is necessary and depends upon Rho GTPases to create membrane extensions, but in this case, multiple smaller particles may be taken up in a bolus of liquid. Dendritic cells are particularly adept at macropinocytosis, which results in the internalization of comparatively large volumes of fluid from the extracellular milieu (Mellman and Steinman, 2001).

Endocytosis mediated by caveolae and clathrin (Conner and Schmid, 2003) are both receptor mediated and transport only limited quantities of fluids. Caveolin-dependent endocytosis occurs at regions of the membrane rich in cholesterol and sphingolipids. Caveolin associates with cholesterol and itself to coat the surface of the invaginated membrane. Clathrin forms a quasicrystalline coat on the inner surface of the plasma membrane that invaginates into pits and then buds to form a coated vesicle. These vesicles quickly uncoat, allowing fusion with endosomes.

Vesicles formed at the membrane fuse with early endosomes or recycling endosomes. The early endosomes act to sort cargo and have a mildly acidic pH. In this compartment, ligands are separated from their receptors, allowing the receptors to cycle back to the cell surface (Maxfield and McGraw, 2004). Subsequently, endocytosed cargo is sorted to the late endosomes. The late endosomes, also called multivesicular bodies because they contain numerous intraluminal vesicles of cytoplasmic material that have budded from the endosomal membrane, are more acidic and are the final sorting location before delivery to lysosomes (Mellman, 1996). Delivery of endosomal contents to lysosomes occurs either through transient or stable membrane fusions of late endosomes and lysosomes (Pryor and Luzio, 2009).

Intracellular TLR Ligand Recognition

The precise molecular interactions between TLRs and their ligands have been determined in a number of cases. TLR specificity is sufficiently relaxed to permit accommodation of numerous LPS isoforms and is perhaps enforced as much by a proscription against recognition of host molecules rather than inflexible rules concerning ligand structure (Beutler et al., 2006). At times, endogenous ligands *are* recognized by TLRs or by TLR coreceptors such as CD36 (Hoebe et al., 2005), and these ligands may conceivably act as danger signals in the setting of tissue damage (Miyake, 2007). As discussed below, some TLRs do not seem to discriminate between molecules of host and microbe origin at all and respond to either one should an encounter occur.

TLR1, TLR2, TLR4, TLR5, TLR6, and probably TLR11 and TLR12 of mice and TLR10 of humans are expressed largely on the cell surface where they recognize molecules derived from microbes of many types (Kawai and Akira, 2009; Kumar et al., 2009). The intracellular TLRs, including TLR3, TLR7, TLR8, and TLR9, are intrinsically capable of detecting nucleic acids. To distinguish between host and foreign nucleic acids, these TLRs act within the endosomal compartment, from which host DNA is usually (but not always) excluded and do so at a particular stage of endosome maturation and acidification. In mice, TLR13 is believed to be located within endosomes because it has been shown to associate with UNC93B1 (Brinkmann et al., 2007), but its ligand remains unknown.

There is a strong sense that the intracellular TLRs function primarily to detect viruses, although it has been shown that they detect other microbes as well. Many viruses are perceived only by endosomal TLRs, and the endosomal TLRs are particularly important to their containment. As discussed above, uptake of intact microbes into the endocytic pathway may occur by receptor-mediated endocytosis, phagocytosis, or nonspecific fluid phase endocytosis. Alternatively, viruses may fuse with the plasma membrane and later be swept into the endosomes either before or during the process of replication as a result of autophagy (Lee et al., 2007). Fully replicated viruses may bud into the endosomal system and traffic through the endosomes on their way to being released from the cell (Wang et al., 2006b).

TLR9 was originally found to detect bacterial DNA. Its specificity was initially thought to be directed toward CpG motifs, which are four times less abundant (and mostly methylated) in mammalian genomic DNA than in bacterial or viral DNA. Thus, TLR9 recognizes dsDNA viruses such as mouse cytomegalovirus (MCMV) and herpes simplex viruses 1 and 2 (Krug et al., 2004a, 2004b; Lund et al., 2003) and the genomes of protozoa, including Trypanosoma cruzi (Bafica et al., 2006). Although malarial DNA is by itself only weakly stimulatory to TLR9, it induces strong TLR9 responses when delivered to the endosomal compartment with a transfection reagent or when bound to malarial hemozoin (Parroche et al., 2007). Thus, hemozoin appears to be a carrier that directs the parasitic DNA to the proper compartment for detection, consistent with the rule that ligand delivery is an essential condition for recognition by the intracellular receptors.

Synthetic CpG oligonucleotides (ODN) containing phosphorothioate linkages stimulate innate immune responses in a TLR9-dependent manner and are commonly used in experimental systems to study TLR9 responses. While there appear to be specific sequences or modifications that optimize the response to nucleic acid ligands (Wagner, 2008), recent work challenges the dogma that unmethylated CpG motifs constitute the foreign signature that triggers TLR9. In fact, it has now been shown that DNA recognition by TLR9 depends on the 2′ deoxyribose phosphate backbone and that neither phosphorothioate linkages nor specific sequences (i.e., CpG motifs) are necessary to induce a response (Haas et al., 2008; Yasuda et al., 2006). Moreover, if nondenatured vertebrate DNA is delivered to the endosomal pathway using a liposome transfection reagent, it can stimulate TLR9 (Yasuda et al., 2005). Phosphorothioate linkages and CpG motifs in synthetic ligands likely increase the stability, aggregation, and uptake of the DNA by cells but are not necessary for interaction with TLR9. Thus, it is ultimately localization to the endolysosomal compartment rather than the sequence, species origin, covalent modification, or double- versus single-strandedness of DNA that dictates TLR9 activation (see below).

TLR7 recognizes ssRNA, imidazoquinoline derivatives such as resiquimod (R848) and imiquimod, and guanine analogs such as loxoribine (Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004). In particular, TLR7 recognizes guanosine- and uridine-rich ssRNA, and synthetic polyuridines act as potent ligands. Through TLR7, the host detects ssRNA viruses, including human immunodeficiency virus (HIV), influenza, and vesicular stomatitis virus (VSV). The host can also utilize TLR7 to detect *Borrelia burgdorferi* and RNA released into endolysosomes by phagosomal bacteria (Eberle et al., 2009; Mancuso et al., 2009; Petzke et al., 2009). Finally, TLR7 may recognize some siRNAs (Hornung et al., 2005). Humans, but not mice, also detect ssRNA through TLR8 (Jurk et al., 2002). And TLR7 (and/or TLR8) is responsible for the detection of host ribonucleoprotein complexes, sometimes with pathologic consequences as described below.

TLR3 is specific for dsRNA and also recognizes the synthetic analog polyinosinic-polycytidylic acid (poly I:C). The dsRNA ligand may be derived from the genomes of dsRNA viruses, for example reovirus. Surprisingly, it was found that TLR3 is additionally able to recognize some ssRNA viruses, including West Nile virus, respiratory syncytial virus (RSV), and encephalomyocarditis virus (EMCV), and even dsDNA viruses such as herpes simplex virus and mouse cytomegalovirus (MCMV) (Tabeta et al., 2004; Wang et al., 2004; Zhang et al., 2007) are sensed via TLR3 responses. During infection, replication of positive sense ssRNA viruses proceeds through a dsRNA intermediate, and dsRNA may also be produced as a result of bidirectional transcription of the genomes of dsDNA viruses. This dsRNA may then be detected by TLR3 (Weber et al., 2006), resulting in some redundancy in sensing of RNA viruses through TLR7 and TLR3, and of DNA viruses through TLR9 and TLR3. Diversity in the signaling pathways triggered by TLR3 versus TLR7 and TLR9 may also allow for some selectivity in the responses that occur after infection by different types of viruses.

Trafficking of Intracellular TLRs

TLR3, TLR7, TLR8, TLR9, and probably TLR13 of mice are expressed intracellularly within the endoplasmic reticulum (ER), endosomes, multivesicular bodies, and lysosomes. However, activation only occurs within acidified endolysosomal compartments, because TLR3, TLR7, TLR8, and TLR9 responses are abrogated by bafilomycin A1, chloroquine, or ammonium chloride, which prevent acidification (Häcker et al., 1998). Based on microscopy studies and the finding that carbohydrates on mature TLR9 lacked the posttranslational modifications typically acquired in the Golgi, it was initially proposed that the intracellular TLRs are ER resident and are quickly translocated to the endolysosomes only upon activation (Latz et al., 2004; Leifer et al., 2004). However, this model raised questions about the nature of the atypical trafficking route utilized by these TLRs and about how cells sense nucleic acids before the intracellular TLRs are mobilized to an acidified endosomal compartment. Analysis of the carbohydrates on endosomal TLR7 and TLR9 has since shown that the receptors must traverse the Golgi

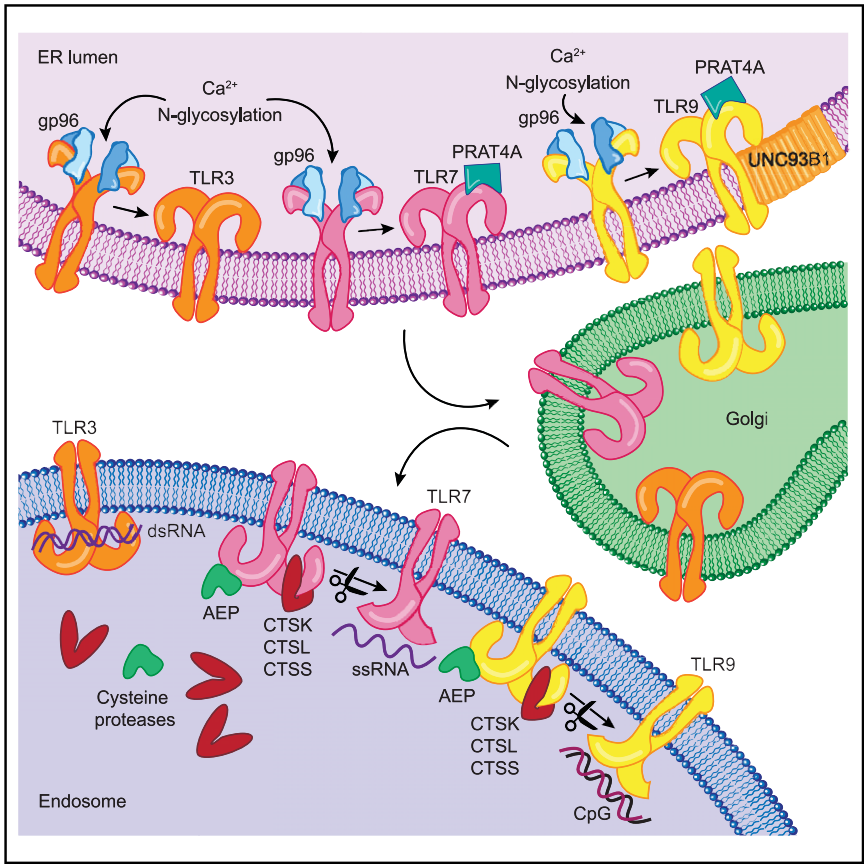

Figure 2. Trafficking and Processing of Intracellular TLRs

Within the endoplasmic reticulum (ER) lumen, gp96 promotes the proper folding of TLR3, TLR7, and TLR9. Gp96, a chaperone similar to Hsp90, functions as a V-shaped dimer that requires intact ER Ca^{2+} stores and N-glycosylation for TLR interaction; ATP hydrolysis (not depicted) is dispensable for binding the TLR but may be necessary for its release. Both PRAT4A and the 12 transmembrane-spanning UNC93B1 are required for TLR3, TLR7, and TLR9 to exit the ER, although their precise functions remain unknown. TLR7 and TLR9 compete for binding to UNC93B1, which normally binds preferentially via an N-terminal region to TLR9. The TLRs traffic through the Golgi by the conventional secretory pathway and are routed to endosomes where they can encounter their ligands. TLR7 and TLR9 are cleaved within their ectodomains by lysosomal cysteine proteases, including the papain-related cathepsins (CTS) K, L, and S, and asparagine endopeptidase (AEP), a protease related to the caspase family and the bacterial endopeptidase gingipain. Cleavage of TLR7 and TLR9 is not strictly required for ligand interaction but is essential for interaction with MyD88 and subsequent signaling. Cleavage of TLR3 has not been observed.

apparatus, revealing that intracellular TLRs traffic from the ER via the common secretory pathway through the Golgi to take up residence in the endolysosomes prior to stimulation (Chockalingam et al., 2009; Ewald et al., 2008; Park et al., 2008).

The intracellular localization of TLR3 is dictated by a region between the Toll/IL-1 receptor (TIR) domain and the transmembrane domain, while intracellular localization of TLR7 and TLR9 depend upon their transmembrane domains. TLR3 is located predominantly within the early endosomes, with cell-surface expression observed in a few cell types (Funami et al., 2004; Matsumoto et al., 2003). Although TLR3, TLR7, and TLR9 can share the same intracellular compartment (Nishiya et al., 2005), it has not been excluded that some compartments contain more restricted TLR subsets and perhaps even single types of these TLRs. Much of the work on localization and function of intracellular TLRs has been focused on TLR9 under the presumption that the rules governing TLR7 and TLR3 localization will be similar, yet this remains to be tested.

Several chaperone proteins associate with TLRs in the ER and are required for efficient translocation (Figure 2). Gp96 (also known as Grp94), the ER paralog of the Hsp90 family, acts as an ER chaperone for IgGs, some integrins, and TLRs. Macrophages lacking gp96 do not respond to ligands for TLRs 1, 2, 4, 5, 7, or 9 (Randow and Seed, 2001; Yang et al., 2007a). N-glycosylation of gp96 and intact ER Ca^{2+} stores are both required for the TLR9-gp96 interaction (Yang et al., 2007a). PRAT4A (also known as Cnpy3) traffics TLR1, TLR2, and TLR4 from the ER to the plasma membrane and TLR7 and TLR9 from the ER to the endolysosome but does not appear necessary for TLR3 trafficking (Takahashi et al., 2007).

Using a forward genetic screening approach, the *3d* ("triple defect" of nucleic acid sensing) mutation was detected and homozygotes were seen to be incapable of responding to ligands for TLR3, TLR7, and TLR9. The *3d* phenotype was ascribed to a mutation in *Unc93Bb1*, a gene encoding a twelve membrane-spanning protein residing chiefly in the ER (Tabeta et al., 2006). UNC93B1 associates with TLR3, TLR7, TLR9, and TLR13 as a result of direct interaction with their transmembrane domains and mediates translocation of these intracellular TLRs from the ER to the endolysosomes (Brinkmann et al., 2007; Kim et al., 2008). TLR7 and TLR9 compete for association with UNC93B1 (Fukui et al., 2009), while TLR3 appears unaffected by this rivalry. Under normal circumstances, UNC93B1 associates more strongly with TLR9, favoring its translocation while biasing against translocation of TLR7. This results in more robust signaling through TLR9 and weaker TLR7 responses. Indeed, overexpression of TLR9 inhibits TLR7 signaling (Wang et al., 2006a). This preference for TLR9 is mediated by an N terminal domain in UNC93B1, and an *Unc93b1* deletion mutant lacking this N terminal domain associates more tightly with TLR7 and less tightly with TLR9. TLR3 appears to translocate to the endosomal compartment with equal efficiency irrespective of the presence or absence of the N-terminal domain that mediates preference for TLR9. Thus, UNC93B1 is ultimately required for intracellular TLR responses and also determines how efficiently each TLR is able to move from the ER to the endolysosomes where they encounter and respond to their respective ligands.

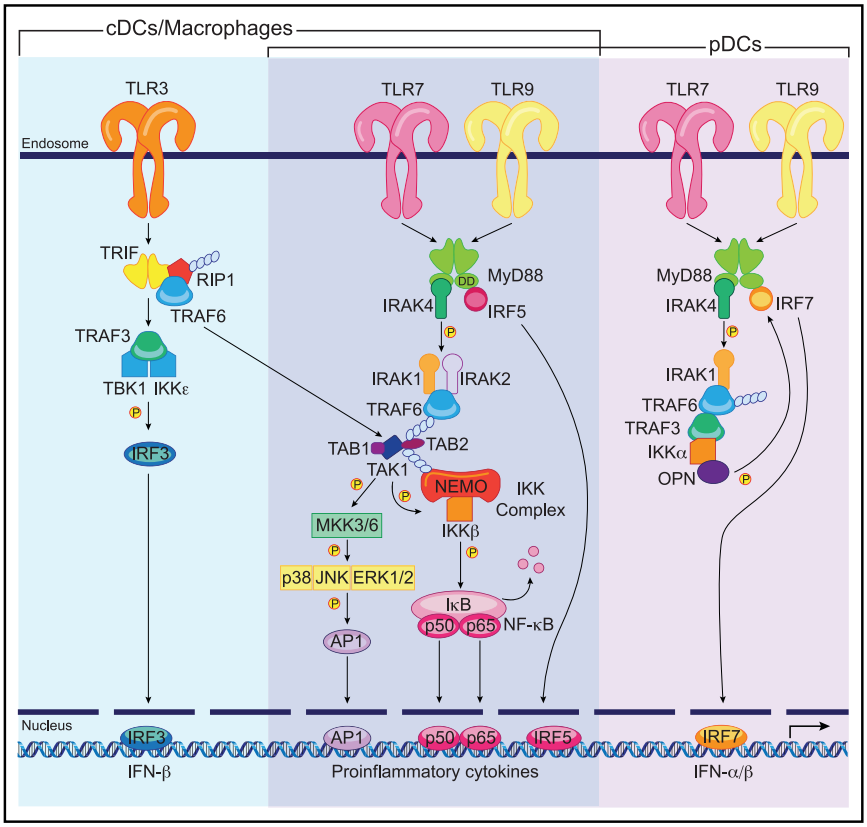

Figure 3. Intracellular TLR Signaling Pathways

TLR3, TLR7, or TLR9 activation by nucleic acids within endolysosomes initiates distinct and overlapping signaling cascades in cDCs, macrophages, and pDCs. Ligand binding to the LRRs of TLRs is thought to induce a conformational change that allows homophilic binding between the TIR domains of receptors and the adapters MyD88 or TRIF. To elicit proinflammatory cytokines (center), MyD88 recruits IRAK4 through death domain interactions. IRAK4 phosphorylates IRAK1 and IRAK2 that, in turn, activate the E3 ubiquitin ligase TRAF6, which together with UBC13 and UEV1A (not depicted), polyubiquitinates NEMO and itself. TRAF6 also activates TAK1. The TAK1-associated TAB2 protein serves as a receptor for the polyubiquitin chains on TRAF6 and NEMO and holds these proteins together. TAK1 phosphorylates IKKβ leading to IκB degradation and release of NF-κB. TAK1 also activates MAPKK3 and MAPKK6, resulting in phosphorylation of JNKs, p38, and CREB. The IKK complex can also phosphorylate p105, which undergoes degradation, leading to the activation of MAP3K8, MKK1, MKK2, ERK1, ERK2, and JNK, culminating in activation of AP1 (not depicted). IRF1 and IRF5 become activated following direct association with MyD88. IRFs, NF-κB, and AP-1 transcription factors induce transcription of proinflammatory cytokines, such as TNF and IL-12. In cDCs and macrophages, signaling from TLR3 (left) induces IFNβ through TRIF, TRAF6, and RIP1, a complex that associates with TRAF3 to activate TBK1 and IKKε, which, in turn, phosphorylate IRF3. Upon TLR3 activation, TRAF6 and RIP1 can also activate NF-κB and MAPKs to induce proinflammatory cytokines (not depicted). pDCs utilize a distinct pathway to induce large amounts of type I IFN (right). Activation of TLR7 or TLR9 in pDCs recruits MyD88 and IRAK4, which then interact with TRAF6, TRAF3, IRAK1, IKKα, osteopontin (OPN), and IRF7. IRAK-1 and IKKα phosphorylate and activate IRF7, leading to transcription of interferon-inducible genes and production of type I IFN.

As already mentioned, endosome acidification is required for TLR3, TLR7, and TLR9 activation by purified ligands (Häcker et al., 1998). Endosome maturation may in some cases facilitate disassembly of microbes in order to release the ligands contained within them but may also contribute to essential processing of the TLRs themselves. For example, upon reaching the endolysosomal compartment, the TLR9 ectodomain is proteolytically cleaved by proteases (Figure 2) (Ewald et al., 2008; Park et al., 2008). Thus, full-length TLR9 is found only in the ER, whereas the cleaved, biologically active product is restricted to endolysosomes. Multiple proteases have been implicated in TLR9 cleavage, including various cathepsins and asparagine endopeptidase (Asagiri et al., 2008; Ewald et al., 2008; Fukui et al., 2009; Matsumoto et al., 2008; Park et al., 2008; Sepulveda et al., 2009), but it is likely that there is at least some redundancy with respect to protease utilization, and the choice of protease may be dependent on cell type. Although not essential, cleavage increases binding to CpG DNA. TLR9 cleavage is required for the recruitment of MyD88 and initiation of signal transduction. Thus, cleavage appears to be an additional step of compartmental regulation limiting TLR-mediated signaling to a specific class of endosomes. Although similar cleavage of TLR7 has been reported by Ewald et al. (Asagiri et al., 2008; Ewald et al., 2008; Fukui et al., 2009; Matsumoto et al., 2008; Park et al., 2008), no cleavage of TLR3 has been observed. Postcleavage forms of TLR7 and TLR9 may take on a structure similar to the ligand binding solenoid shape of full-length TLR3 (Bell et al., 2005; Ewald et al., 2008; Liu et al., 2008).

TLR Signaling

All TLRs, with the exception of TLR3, depend at least in part upon the MyD88 adaptor protein for full signaling activity, and some TLRs (including TLRs 7 and 9) are completely dependent upon MyD88. Ligand binding to the leucine rich repeats (LRRs) in the ectodomain induces a change in the conformation and/or association of TLR ectodomains that is believed, in turn, to affect the association of the cytoplasmic TIR domains (Latz et al., 2007), eliciting a signal. Signaling entails association between TIR domains of the TLRs and TIR domains of adaptor molecules, but no crystallographic data have yet permitted a detailed understanding of these events. Certain mutations in MyD88 produce receptor-selective effects (Jiang et al., 2006), and while some parts of the MyD88 TIR domain are important to permit its recruitment to a specific TLR, other parts are required for propagation of the signal downstream (Dunne et al., 2003; Li et al., 2005). MyD88 contains an N-terminal death domain (DD), which interacts with IL-1R-associated kinase-4 (IRAK-4) through its own DD (Figure 3) (Suzuki et al., 2002). IRAK4 phosphorylates IRAK1 and IRAK2 (Kawagoe et al., 2008); they in turn activate TNF receptor-associated factor 6 (TRAF6), which is inhibited

by another IRAK family member, IRAK-M (Kobayashi et al., 2002). TRAF6, an E3 ubiquitin ligase, combined with UBC13 and UEV1A forms polyubiquitin chains on NF-κB essential modulator (NEMO or IKKγ) (Siggs et al., 2010; Skaug et al., 2009) and on itself. TRAF6 also activates the MAPKKK member TGF-β activated kinase 1 (TAK1), which associates with two adaptor proteins: TAK1-binding proteins 1 (TAB1) and TAB2. TAB2 serves as a receptor for the polyubiquitin chains on TRAF6 and NEMO and "glues" these proteins together. TAK1 phosphorylates IKKβ and also activates a cascade of mitogen-activated kinases (MAPKs), starting with MAPKK3 and MAPKK6, leading to phosphorylation of Jun kinases (JNKs), p38, and ultimately, CREB. The IKK proteins phosphorylate IκBs, resulting in their degradation and resultant nuclear translocation of NF-κB. They additionally phosphorylate p105, another member of the NF-κB family, which undergoes degradation, leading to the activation of MAP3K8. MAP3K8, also known as Tpl2, is a protein kinase that activates MKK1 and MKK2, which in turn activate extracellular signal regulated kinases ERK1 and ERK2 and the Jun kinases (JNK), culminating in activation of AP1. The IRF family of transcription factors is also activated. IRF1 associates directly with MyD88 (Negishi et al., 2006), and IRF5 associates with both MyD88 and TRAF6 (Takaoka et al., 2005). Ultimately, activation of IRFs, NF-κB, and AP-1 transcription factors induces gene transcription of proinflammatory cytokines, such as TNF and IL-12.

TLR3 does not signal through MyD88 but instead associates with the adaptor TRIF (Hoebe et al., 2003). TRIF associates with TRAF3 and TRAF6, as well as receptor-interacting proteins 1 and 3 (RIP1 and RIP3). TRAF6 and RIP1, with the help of TRADD and TAK1, activate NF-κB and MAPKs to induce proinflammatory cytokines (Ermolaeva et al., 2008; Pobezinskaya et al., 2008). TRAF3 links TBK1 to the TRIF-dependent pathway (Häcker et al., 2006), which in combination with IKKε, phosphorylates and activates IRF3 (Fitzgerald et al., 2003; Sharma et al., 2003), leading to IFNβ production. TLR4 also signals through the same TRIF pathway but, under most circumstances, additionally requires the adaptor TRAM.

Although TLR3, TLR7, TLR8, and TLR9 are localized and act exclusively from intracellular compartments, other TLRs may, in some instances, also signal from locations within the cell. TLR4 ligand recognition occurs at the cell surface, resulting in recruitment and signaling through MyD88. Subsequently, receptor internalization into early endosomes halts MyD88-dependent signaling and triggers TRIF-dependent signaling (Husebye et al., 2006; Kagan et al., 2008; Tanimura et al., 2008). Finally, TLR4 migrates to late endosomes, where it encounters TAG, a splice variant of TRAM that disrupts the interaction between TRIF and TRAM, arresting TRIF-dependent signaling and ultimately promoting degradation of the signaling complex (Palsson-McDermott et al., 2009). TLR2 is also internalized following ligand binding, but in this case, MyD88-dependent signaling continues from an intracellular location away from the plasma membrane and stimulates type I IFN production through an as yet unknown mechanism (Barbalat et al., 2009). These data suggest that TRIF signals strictly from an intracellular location, whereas MyD88 can signal both when associated with the plasma membrane and from within endosomal compartments.

TLR7 and TLR9 Signaling in Plasmacytoid Dendritic Cells

Plasmacytoid dendritic cells (pDCs) are unique in their ability to couple TLR7 and TLR9 signaling with production of abundant quantities of type I IFN. The signaling events described above occur in macrophages, conventional dendritic cells (cDCs), B cells, and pDCs and lead to production of proinflammatory cytokines, such as TNFα, IL-6 and IL-12, but not type I IFN. In pDCs, however, an additional pathway exists (Figure 3): activation of TLR7 or TLR9 recruits MyD88 and IRAK4, which then interact with TRAF6, TRAF3, IRAK1, IKKα, osteopontin, and IRF7 (Kawai et al., 2004; Shinohara et al., 2006). Ultimately, IRAK-1 and IKKα phosphorylate IRF7, leading to activation, which induces abundant transcription of type I IFN genes and production of enormous quantities of type I IFN (Honda et al., 2005b).

It is presently unclear whether both signaling pathways, one leading to proinflammatory cytokine production and the other to type I IFN production, occur simultaneously or sequentially. Furthermore, it is not understood how and why pDCs are able to utilize this unique pathway to generate large amounts of type I IFN in response to TLR signaling through MyD88. Retention of the signaling complex within the early endosome in pDCs, as opposed to the late endosome or lysosome in other immune cells, appears to correlate with IRF-7 recruitment and IFN induction (Honda et al., 2005a), suggesting that TLR signaling from early endosomes is different in nature than that from late endosomes. The nature of this difference remains unknown but may be due to altered pH or inclusion of additional signaling molecules not present in more mature compartments. Alternatively or in conjunction with this idea, it has been proposed that basal induction of IRF7 in pDCs allows these cells to quickly produce large amounts of type I IFN (Honda et al., 2005b). It could be that other adaptations unique to pDCs have been built around the TLR sensing and signaling apparatus to augment the interferon response.

Two types of synthetic CpG ODN induce differential cytokine responses from immune cells. For pDCs, Type "A" CpG ODN (CpG-A) mainly induce type I IFN, whereas type "B" CpG ODN (CpG-B) predominantly induce proinflammatory cytokines. In structural terms, CpG-B contain single or multiple CpG motifs on a phosphorothioate backbone. These CpG-B molecules remain as single-stranded linear monomers, allowing them to traffic rapidly to the endosome and lysosome (Guiducci et al., 2006; Honda et al., 2005a). On the other hand, CpG-A contain a single CpG motif and a 3′ poly-G tail on a mixed phosphorothioate-phosphodiester backbone, which mediate aggregation of CpG-A resulting in a longer retention time in the early endosome (Kerkmann et al., 2005). It is thought that this prolonged period in the early endosome results in extended activation of the MyD88-TRAF6-IRAK1-IRF7 complex and thereby promotes robust type I IFN production, as discussed above. Differential responses of pDCs to CpG-A and CpG-B were thus proposed to stem from detection of CpG-A and CpG-B in distinct compartments. However, it is unclear whether CpG ODN trafficking conforms to the physiological trafficking pathways for microbial molecules. Viral nucleic acids likely stimulate cells in a manner more similar to CpG-A, as has been shown for influenza virus stimulation of TLR7 (Di Domizio et al., 2009). Thus, the

importance of differential responses to CpG-A and CpG-B ODN and the implications concerning responses to DNA derived from infectious agents remain ambiguous, although it is clear that pDCs are specialized for the generation of abundant quantities of type I IFN in response to both artificial and microbial ligands for TLR7 and TLR9.

By comparing the signaling pathways utilized by cell-surface TLRs with those of intracellular TLRs, as well as other intracellular receptors, a unifying picture emerges that the location of the receptor dictates the signal produced. Type I IFN is produced solely by recognition of ligands present in the cytosol or within the lumen of intracellular compartments. In part, this reflects the compartmentalization of signaling adaptors and proteins. The most striking example of this is TRAF3, which is the only signaling component shared by all the interferon-inducing signaling pathways (Saha and Cheng, 2006). Notably, signaling that elicits type I IFN production through the TLR3 → TRIF axis, TLR4 → TRIF axis, or the TLR2 → MyD88 axis probably occurs within early endosomal compartments. Further studies will establish whether the early endosome indeed represents the sole compartment from which type I IFN may be induced by TLR signaling.

Compartment-Based Ligand Recognition Is Key to Distinguishing Self from Foreign Nucleic Acids

Nucleic acid sequences are the targets of choice for sensing viruses. Nucleic acids are critical viral components present throughout the viral life cycle. Viral nucleic acids display features that permit discrimination from host nucleic acids, including long dsRNA, 5′-triphosphate RNA, and unmethylated CpG DNA, which can serve as triggers for innate immune receptors. Nonetheless, physical separation of host nucleic acids from foreign nucleic acids and the intracellular receptors that detect them is the key to distinguishing self from invader.

Proper trafficking of TLR7, TLR8, and TLR9 is essential to prevent receptor activation by self DNA, leading to autoimmunity. Host DNA introduced into the endosomal compartment using a liposomal transfection reagent can stimulate TLR9 (Yasuda et al., 2006). Conversely, forced expression of TLR9 at the cell surface permits cells to bind self DNA within the extracellular space and blocks sensing of viral DNA (Barton et al., 2006). Thus, altering receptor compartmentalization would be predicted to permit access to host DNA within the extracellular milieu while preventing access to endosome-protected viral ligands. It is now known that TLR9 cleavage is required for efficient ligand binding and association with MyD88. It is not clear how a surface-directed TLR9 complex would undergo processing to make it competent to signal. Potentially, ectopically expressed TLR9 might cycle or recycle through endosomal compartments, allowing cleavage to occur.

Under normal circumstances, host DNA is sequestered in nuclei or mitochondria, and extracellular and endosomal DNases and RNases hydrolyze free nucleic acids, preventing inappropriate activation of the innate immune system. Accordingly, nuclease-resistant phosphorothioate-stabilized or 3′-polyguanosine oligonucleotides are especially strong activators of TLR9 (Haas et al., 2008; Yasuda et al., 2006). Special mechanisms may normally shield sensing systems of the cell from DNA during apoptosis, when it might otherwise be exposed to innate immune receptors as the nuclear membrane breaks down. DNA is most likely degraded as the cell is transformed into apoptotic bodies. In this way, cells and their endogenous TLR ligands are silently eliminated.

Intracellular Nucleic Acid Sensing and Autoimmunity

Nucleic acid sensing pushes self-nonself discrimination to its limits and has come to depend upon physical separation of host nucleic acids from the innate immune receptors that would otherwise detect them. Autoimmune or autoinflammatory diseases develop when self DNA comes into contact with intracellular TLRs or other cytoplasmic nucleic acid sensors. Activation of intracellular TLRs, in particular TLR7 and TLR9, have been implicated in the production of autoantibodies to nuclear and cytoplasmic material containing RNA and/or DNA in mice with systemic lupus erythematosus (SLE)-like disease (Marshak-Rothstein, 2006). In lupus-prone BXSB mice, the Y-linked accelerator of autoimmunity (*Yaa*) locus encodes an extra copy of TLR7 on the Y chromosome, a circumstance that markedly accelerates the progression of a lupus-like disorder. *Yaa* has a general effect, promoting SLE progression in other lupus-prone strains as well. Sequential engagement of cell surface BCR and endosomal TLRs is necessary and sufficient for autoantibody production. B cell autoreactivity may also be promoted by nucleic acid sensing in accessory cells. For example, it has been shown that interplay between pDCs and B cells is required for full activation of autoreactive B cells upon TLR or BCR stimulation (Ding et al., 2009).

Untoward encounters between host nucleic acids and host nucleic acid sensors may occur when the former escape degradation during programmed cell death. Dysfunction of Flap endonuclease 1 (FEN1) has been shown to result in residual undigested self DNA within apoptotic bodies leading to chronic inflammation and autoimmunity (Zheng et al., 2007). Deficiencies in extracellular DNase I results in a SLE-type syndrome in mice (Napirei et al., 2000). Additionally, human SLE patients exhibit lower serum DNase I activity and some mutations within the DNase I gene have been identified (Yasutomo et al., 2001). Dysfunction of lysosomal DNase II also results in inadequate degradation of DNA, leading to a variety of autoimmune syndromes including arthritis and anemia (Kawane et al., 2006; Okabe et al., 2005; Yoshida et al., 2005). The Trex1 endonuclease mediates DNA degradation during granzyme A-mediated cell death, and mutations of human TREX1 result in SLE, familial chilblain lupus, inflammatory myocarditis, and Aicardi-Goutieres syndrome (Crow et al., 2006; Lee-Kirsch et al., 2007a, 2007b). In mice, targeted deletion of *Trex1* results in reduced lifespan and inflammatory myocarditis and cardiomyopathy by 2–4 months of age (Morita et al., 2004). An IRF-3-dependent type I IFN response was shown to be required for development of inflammatory myocarditis. In Trex1-deficient cells, 60–65 bp ssDNAs accumulate in the ER, leading to activation of the ataxia telangiectasia mutated (ATM)-dependent DNA damage checkpoint (Yang et al., 2007b). Cloning of the accumulated Trex1 substrates suggests that Trex1 may degrade reverse transcribed DNA arising from endogenous retroelements (Stetson et al., 2008).

Complexing of host proteins and nucleic acids may prevent degradation of nucleic acids as it normally occurs or may permit

nucleic acids to be taken up by receptor-mediated endocytosis. Higher concentrations of nucleic acid ligands, shorter transit times, trafficking by a different route, or longer retention times within the endosomal compartment may also result. TLR9 responses in B cells are triggered following BCR-mediated uptake of proteins complexed to nucleic acids (Lau et al., 2005; Leadbetter et al., 2002). The antimicrobial peptide LL37 released during skin injury binds to self DNA released during tissue damage and creates aggregates that enter the endosomal pathway and are retained there. Although this likely boosts resistance to infection under normal circumstances, when LL37 is aberrantly expressed, it can result in autoimmunity manifesting as psoriasis (Lande et al., 2007). Anti-nuclear antibodies may form complexes with self DNA-bound nuclear proteins if tolerance to nuclear components is lost, thereby facilitating FcR mediated self DNA uptake into endosomes where it can activate TLR signaling (Boulé et al., 2004).

Perspectives

Many essential questions regarding intracellular innate sensing remain unanswered. First, we would like to know the essential biochemical distinctions between intracellular and cell-surface signaling and their structural bases. And these distinctions may be exceptionally complex, insofar as the machinery built around TLRs differs from one cell type to another. Witness the difference in signaling in macrophages as compared to pDCs for example. Comparable differences—or perhaps greater ones—may distinguish TLR signaling in other cells types (e.g., epithelial cells, neurons, and fibroblasts) where TLRs certainly exist and do respond to ligands but have been less studied than they have been in immune cells.

Although much progress has been made in recent years, mechanistic details regarding trafficking of intracellular receptors, their ligands, and associated signaling molecules remain unclear. A few of the molecular requirements (e.g., UNC93B1 and gp96) are known, but many more remain to be defined, and we are far from a detailed understanding. One pathway to progress is forward genetic analysis of the TLR response (Beutler and Moresco, 2008). Screens of ENU mutagenized animals have disclosed components of the signaling apparatus that might otherwise have escaped notice. Subsequent placement of these components within pathways and decipherment of their modes of action has allowed some of the most surprising advances in the field. The forward genetic strategy could be used to specifically probe intracellular recognition. A screen for impaired production of type I IFN, conducted in pDCs activated by TLR7 or TLR9 or macrophages stimulated with TLR3, might be entertained for this reason. Similarly, screens designed to detect the impairment of RLH and NLR signaling could readily be exploited to find new molecules. As essential molecules are identified, we may hope to entertain hypotheses about their precise relationship to one another, and their roles in self-nonself discrimination.

In conclusion, the intracellular TLRs represent a unique system by which infection is detected through recognition of nucleic acids. Differential compartmentalization of TLRs dictates not only which ligands are recognized, but also the nature of the signals generated. Thus, a complex regulatory system exists to ensure that both ligands and receptors are properly localized. Ultimately, a delicate balance exists whereby deviations can result in immune deficiency on one side or autoimmunity on the other.

ACKNOWLEDGMENTS

We are grateful to E.M. Moresco for her critical reading of this paper and assistance in its preparation. This work was supported by NIH grant P01 AI070167 and NIH Contract HHSN272200700038C. A.L.B. is supported by The Irvington Institute Fellowship Program of the Cancer Research Institute.

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